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Elevated CO_2, chlorpyrifos and biochar influence nitrification and microbial abundance in the rhizosphere of wheat cultivated in a tropical vertisol

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ABSTRACT

Climate change is predicted to increase pest infestation and may lead to high use of insecticides in agriculture. Elevated CO_2 and other climate factors affect soil microbial processes like nitrification. To regulate climate change biochar (BC) has been recommended for soil application. It is hypothesized that elevated CO_2 and biochar may stimulate autolithotrophic nitrifiers and providing a matrix for microbial proliferation while chlorpyrifos inhibit nitrification through toxicity effect on soil microorganisms. To validate these hypotheses, Experiments were carried out to estimate nitrification in rhizosphere of wheat under the influence of elevated CO_2, chlorpyrifos and biochar. The experimental factors were CO_2 (400 ppm, 800 ppm), biochar (0%, 1%), and chlorpyrifos (0 ppm, 10 ppm). The parameters were nitrification rate, abundance of 16S rRNA gene of eubacteria, abundance of amoA gene, plant shoot and root biomass. Nitrification rate and microbial parameters were lowest in the treatment of CO_2 400 ppm biochar 0% chlorpyrifos 10 ppm and high under CO_2 800ppm biochar 1% chlorpyrifos 0 ppm. Chlorpyrifos inhibited nitrification rates by 3 folds. Similarly, the abundances of 16S rRNA gene copies of eubacteria, ammonia oxidizing bacteria were inhibited 1.36 and 2.68 times by chlorpyrifos. Plant parameters (shoot and root biomass) were highest under elevated CO_2 biochar 1% and chlorpyrifos 0 ppm and lowest in CO_2 400 ppm biochar 0% and chlorpyrifos 10 ppm. Principal component analysis denoted that PC1 contributed 93.28% variation and PC2 contributed 4.40% variation. Study concludes that chlorpyrifos inhibit nitrification while elevated CO_2 and biochar may alleviate these negative impacts aiding in retaining soil function.

1. Introduction

The commonly known three major greenhouse gas (GHG) in the atmosphere are CO_2, CH_4 and N_2O. Among the three GHGs the most rapidly rising one is CO_2. The atmospheric CO_2 concentration is increasing alarmingly at 3% per year and it is predicted that even with stringent policies to curb GHG emission, the concentration of CO_2 will reach 550–700 ppm by 2050 and 650–1200 ppm by 2100 (Higgins et al., 2015).

Elevated CO_2 and temperature increases atmospheric moisture content and will intensify the prevalence of pests. Therefore, climate change will intensify the use of insecticides in agriculture (Yan et al., 2017). One of the most widely used insecticide is chlorpyrifos (0,0-diethyl-3,5,6-trichloro-2- pyridylphosphorothioate) (Gomez, 2009). Elevated CO_2 and temperature influences the persistence of chlorpyrifos in agricultural soil (Ahirwar et al., 2018). Probably, intensive use of chlorpyrifos under climate changing condition may affect soil biological function severely due to longer persistence.

Biochar is recommended to improve carbon sequestration and mitigate GHG emission. It can enhance plant growth by improving nutrient use efficiency (Kollah et al., 2015). Biochar is prepared by pyrolysis of organic feedstock or biomass (Lehmann, 2007). It has very high cation exchange capacity (CEC) enabling it to absorb NH_4-N and other plant nutrients (Subedi et al., 2013). Biochar adsorbs insecticides and reduce toxicity to the plants (Diez et al., 2013). Biochar stimulates insecticide biodegradation by stimulating heterotrophic microbial groups (Atkinson et al., 2010).

Nitrification is one of the important soil biological functions in the prospect of plant nutrition, climate change and global nitrogen balance. Nitrification is a two-step process performed by two distinct microbial groups (Alfreider et al., 2017). The first microbial group oxidize NH_4 to NO_2, and the second oxidize NO_2 to NO_3 (Weber et al., 2015). Wheat is

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cultivated widely in the temperate and tropical countries. Moreover, this crop requires intensive input of N fertilizer and insecticide. It is hypothesized that nitrification in the rhizosphere of wheat is influenced by elevated CO_2 and inhibited by chlorpyrifos. Biochar may alleviate the negative impact of the insecticide to a great extent in soil ecosystem. Experiments were carried out to evaluate the influence of elevated CO_2, chlorpyrifos and biochar on nitrification and soil microorganisms in the rhizosphere of wheat grown in a vertisol.

2. Materials and methods

2.1. Soil sampling site

Soil samples were collected from an experimental field of the Indian Institute of Soil Science, Bhopal, Madhya Pradesh, India (23.30 N, 77.40 E, 485 m asl). The experimental field was cultivated with soybean (*Glycine max* L.) during summer and wheat (*Triticum aestivum* L.) during winter since 2004. Wheat (variety HI 8498) and soybean (variety JS 335) were grown at a spacing (cm) of 22.5 × 5 and 45 × 5 respectively, with seeding rates of 100 kg and 80 kg ha^{-1} respectively. Soils were collected from the control plot that received no fertilizer. Soil sampling was done during June 2017 when there was no crop. A composite sample was prepared by mixing 4 samples from corners and 1 sample from centre of the field. Sampling was done from ploughing depth profile (5–15 cm). The location has a humid subtropical climate, with a hot summer and a humid monsoon season. It experiences south-western monsoon rains between July and September. Mean annual temperature remains about 25 °C. Highest temperature reaches ~45 °C during the mid summer (May–June). During winter (December–January) the mean temperature remains about 15 °C, the average yearly precipitation is 1200 mm and air humidity remains ~65%. After collection from field, the soils were hand-processed by breaking the clods and removing roots and stones. Soil was then passed through a 2-mm mesh sieve and used within 2 days of collection.

2.2. Soil physico-chemical properties

The soil is a heavy clayey Vertisol (Typic Haplustert) contained 5.7 g organic C, 225 mg available N, 2.6 mg available P, and 230 mg available K per kg soil. Organic carbon (OC) was estimated by wet digestion method (Walkley and Black, 1934). Available N was determined by alkaline $KMnO_4$ method (Subbiah and Asija, 1956). Available P was extracted by 0.5 N $NaHCO_3$ pH 8.5 (Olsen, 1954). Available K was extracted by neutral normal CH_3COONH_4 (Hanway and Heidel, 1952) and determined by a flame photometer (Lindsay and Norvell, 1978). The electrical conductivity (EC) was 0.43 dS m^{-1} and the pH was 7.5 (1:2.5 of soil and water in w:v) (Smith and Doran, 1996). The water holding capacity, bulk density and saturated hydraulic conductivity of the soil were 62% (w/w), 1.45 mg m^{-3}, and 7.3×10^{-6} m s^{-1} respectively. The textural composition of soil was: sand 15.2%, silt 30.3%, clay 54.5%. The field soil had 863.24 µM NO_3^-, 0.01 µM Fe^{2+} and 101.02 µM SO_4^{2-}.

2.3. Biochar preparation

Biochar (BC) was prepared from the stalks of pigeon pea (*Cajanus cajan*) grown in the experimental farm of Central Institute of Agricultural Engineering Institute, Bhopal, India adjacent to the Indian Institute of Soil Science, Bhopal. The sun dried of pigeon pea stalks were shredded to 5–7 cm in length. The kiln was an unconfined insulated chamber made of mild steel with dimension: inner diameter 36 cm, height 50 cm and wall thickness 0.2 cm. Chamber temperatures was maintained at 450 °C by external heating (heating rate 6 °C min^{-1}). Charring of pigeon pea stalks was completed in 4 h (Gangil, 2014). The BC was grounded manually and passed through 2 mm sieve for experimental use. Biochar was characterized by standard protocols (Nelson and Sommers, 1982). The pH (1:1.25, H_2O), electrical conductivity, ash content and bulk density were 9.57, 1.95 (dS m^{-1}), 15.5% and 239 kg m^{-3} respectively. The total C, N, P and K concentrations (%) were 86.4, 0.40, 0.09, and 0.74 respectively. Total Ca, Mg, and Na concentration (mg kg^{-1}) was 92.2, 19.5, and 395 respectively.

2.4. Microcosms set-up

The experiment used a factorial design to determine the impact of factors (elevated CO_2, biochar and insecticide) on nitrification. The factors were CO_2 (400 ppm, 800 ppm), chlorpyrifos (0 ppm 10 ppm) and biochar (0%, 1%), Biochar is applied to fields at 1–2% as soil amendment (Asai et al., 2009). Each factorial combination (2 CO_2 × 2 biochar × 2 chorpyrifos) was replicated 3 times, for a total of 18 experimental units. Soil of 5 kg was weighed into pots and mixed with chlorpyrifos stock solution. A 1000 ppm stock solution of chlorpyrifos (Sigma Aldrich, USA) was prepared using HPLC grade acetonitrile (Sigma Aldrich, USA). The chlorpyrifos stock of 0.05 ml mixed with 10 ml water was added to soil to represent 10 ppm (w/w) chlorpyrifos. This concentration is equivalent to 5 kg active ingredients per ha was within the range of field doses recommended for different crops. Similarly, the soil added with 10 ml water containing 0.05 ml pure acetonitrile served as treatment of 0 ppm chlorpyrifos. Biochar was added to pots at the level of none (0%) or 1% (w/w). The soils were also mixed with aqueous stock of NH_4Cl, KCl, and $Ca(H_2PO_4)_2$ for a final concentration of 50 mg NH_4-N, 25 mg K_2O –K, and 25 mg P_2O_5-P per kg soil. After adding all constituents soil was mixed thoroughly. Distilled water was added to maintain 60% moisture holding capacity. Pots were kept in a walk in plant growth chamber (M/s Genesis, Mumbai, India). The plant growth chamber had 2 compartments for setting 2 different CO_2 concentrations (400 ppm, 800 ppm). Wheat (*Triticum aestivum* L, var HI 8498) was sown in each pot (two seed per pot). Plants growth chamber was equipped with CO_2 sensor, red green blue LED tube lights, infrared heater, air conditioner, and microwave humidifier. The plant growth chamber was set for 18 h illumination per day, temperature 25 ± 2 °C and relative humidity 70 ± 5%. At regular interval (2 days) pots were moistened with distilled water to maintain moisture.

2.5. Nitrification measurement

To evaluate nitrification soil samples were collected from pots (3–5 cm below surface) near to root zone. Concentration of NO_3^- was estimated after extracting with $CaSO_4$ and estimated by phenol disulphonic acid method (Jackson, 1958). Potential nitrification rate (PNR) was determined from the increase in concentration of NO_3^{1-}. Soil sampling and NO_3 estimation was carried out for 21 days as the concentration of NO_3 remained un-changed thereafter. The slope of the regression line out of the plot of NO_3^{1-} N vs incubation time represented the potential nitrification rate (µg NO_3^{1-} produced g^{-1} soil d^{-1}) (Schmidt and Belser, 1982).

2.6. DNA extraction

Genomic DNA from soil samples (0.5 g) was extracted using the ultraclean DNA extraction kit (MoBio, USA). Concentration of extracted DNA were measured in a biophotometer (Eppendorf, Germany), assuming 1 absorbance at wavelength 260 nm corresponds to 50 ng of DNA per µl. Quality of extracted DNA was further checked by electrophoresis on a 1% agarose gel. The extracted DNA was stored at −20 °C until further analysis.

2.7. Quantification of 16S rRNA gene of eubacteria and amoA gene of ammonia oxidizing bacteria

The copies of 16S rRNA and amoA genes in soil were quantified by a

real time PCR (Step one plus, Applied Biosystems, USA). Reaction mixture contained 2 µl of DNA template, 10 µl of 2X SYBR green master mix (Affymetrix, USA), 200 nM of primer (GCC Biotech, N Delhi). Final volume of PCR reaction mixture was adjusted to 20 µl with molecular biology grade water. The primers (5′-3′) for eubacterial 16S rRNA gene were 1F (CCT ACG GGA GGC AGC AG) and 518R (ATT ACC GCG GCT GCT GG) (Baek et al., 2010). The primers for nitrifying bacteria were 1F (GGG GTT TCT ACT GGT GGT) and amoA 2R1 (CCC CTC TGG AAA GCC TTC TTC) (Okano et al., 2004). Thermal profile of PCR amplification was as follows: initial denaturizing step (94 °C for 4 min); then 40 cycles of 94 °C for 1 min, annealing temperature for 30 s, 72 °C for 45 s; final extension at 72 °C for 5 min. The annealing temperature for 16S rRNA was 52 °C, and for amoA was 50 °C. Fluorescence was measured during elongation step. Data analysis was carried out with Step one plus software (ABI, USA) following user's manual. The C_T (threshold cycle) values (cycle at which the fluorescence of target molecule number exceeded the background fluorescence) were determined for each targets. The quality of PCR amplification products were examined by a melting curve analysis with temperature increase of 0.3 °C per cycle and data was presented as number of gene copies or cells g^{-1} soil.

2.8. Plant growth parameters

Wheat plants were harvested after 60 days of sowing. Plants were carefully removed from soil with minimal damage to roots. Soil particles adhering to root were washed out with tap water. Plants were cut into root and shoot parts. Biomass of root and shoot was determined.

2.9. Statistical analysis

Results for the experiments were presented as arithmetic means and standard deviation of triplicate observations. Tukeys honestly significant difference (HSD) test was performed to define the significant difference among treatments at α 0.05. Effect of factors (CO_2 concentration, biochar and chlorpyrifos) on the variables or parameters (nitrification, abundance of 16 S rRNA gene of eubacteria, amoA gene of ammonia oxidizers, root biomass and shoot biomass) was tested by analysis of variance (ANOVA) at α 0.05. Estimated data were ln transformed for ANOVA. Linear regression models were developed to predict nitrification from different variables. To elucidate the complex interaction among factors and parameters a principal component analysis (PCA) was performed using ln transformed values. PCA was interpreted graphically by constructing a biplot. The angles of the biplots depict the direction of correlation, while the length depicts the extent of correlation. Biplot was made using the values of three replicated observations. All statistical analyses were carried out using the “agricolae” and “vegan” packages of the statistical software R (2.15.1) (Ihaka and Gentleman, 1996).

3. Results

3.1. Gross nitrification

The initial concentration of NO_3-N in the native soil was 12.25 mg kg^{-1}. About 50 mg NH_4-N (NH_4Cl) kg^{-1} soil was added to study nitrification. The nitrification efficiency of the soil remained within 30–66% among various treatments. Nitrification was studied in terms of NO_3-N produced out of the NH_4-N added. The temporal variation of nitrification in the rhizosphere of wheat under the influence of CO_2, biochar and chlorpyrifos is given in Fig. 1. The NO_3-N measurement was carried out for 21 days as the concentration of NO_3-N stabilized after 16–18 days irrespective of treatments. The highest NO_3-N was observed during 8–10 days. During this period the concentration of NO_3-N varied from 19.09 to 41.5 mg kg^{-1} soil. Apparent nitrification rate was determined accounting the period of increasing NO_3-N. Apparent nitrification rate varied among the treatments (Table 1). Lowest nitrification rate (0.665 mg NO_3-N produced g^{-1} soil d^{-1}) was in the treatment of 400 PPM CO_2 biochar 0% chlorpyrifos 10 ppm. Highest nitrification rate (4.525 mg NO_3 –N produced g^{-1} soil d^{-1}) was in the treatment of CO_2 800 ppm biochar 1% chlorpyrifos 0 ppm. The result indicated that the elevated CO_2 and biochar stimulated the nitrification while chlorpyrifos inhibited the nitrification. Elevated CO_2 stimulated nitrification rate by factors of 1.95 to 2.84 than ambient CO_2 ($p < 0.01$). Biochar stimulated nitrification by factors of 14% to 38% than no biochar ($p < 0.05$). Chlorpyrifos inhibited nitrification by factors of 59% to 67% than no chlorpyrifos ($p < 0.01$).

3.2. Microbial abundance

Abundance of eubacteria and ammonia oxidizing bacterial population was estimated during the peak nitrification phase (Table 1). The gene copies of 16S rRNA of eubacteria varied from $25 \pm 3.00 \times 10^6$ g^{-1} soil to 75 ± 4.04 10^6 g^{-1} soil. Highest number of eubacteria was in the treatment of 800 ppm CO_2 biochar 1% with no chlorpyrifos. Lowest number of eubacteria was in the treatment of 400 ppm CO_2 without biochar and with chlorpyrifos 10 ppm. The abundance nitrifies (amoA gene copies of ammonia oxidizing bacteria) varied in from $8.33 \pm 0.577 \times 10^5$ cells g^{-1} soil to $1.43 \pm 0.404 \times 10^5$ cells g^{-1} soil. Highest number of ammonia oxidizer was in the treatment of 800 ppm CO_2 biochar 1% and no chlorpyrifos. Lowest number of ammonia oxidizer was in the treatment of 400 ppm CO_2 0% biochar and 10 ppm chlorpyrifos. Result showed that both elevated CO_2 and biochar acted as stimulator to eubacteria and ammonia oxidizers. Contrastingly, chlorpyrifos inhibited the proliferation of these microbial groups. Elevated CO_2 stimulated the abundance of eubacteria by factors of 1.35 to 1.58. Similarly elevated CO_2 stimulated the abundance of ammonia oxidizing bacteria by factors of 2–2.50 fold. Biochar stimulated eubacterial 16S rRNA gene abundance at 400 ppm CO_2 by a factor of 1.15 to 1.20 and at 800 ppm of CO_2 by a factor of 1.01 to 1.23. Similarly, biochar stimulated nitrifiers amoA gene abundance at 400 ppm CO_2 by a factor of 1.10 to 2.12 and at 800 ppm of CO_2 b a factor of 1.06 to 1.40. Chlorpyrifos inhibited both groups by 17% to 58% than no chlorpyrifos.

3.3. Plant growth attributes

Shoot biomass varied from 43.0 ± 3.7 g to 23.0 ± 2g while the root biomass varied from 25.4 ± 2.33g to 9.6 ± 0.577g (Table 2). Plant growth (both shoot biomass and root biomass) was highest in the treatment of 800 ppm CO_2 biochar 1% and chlorpyrifos 0%. Lowest growth was observed in the treatment of 400 ppm CO_2 biochar 0% and chlorpyrifos 10 ppm. Elevated CO_2 and biochar stimulated the growth of wheat while chlorpyrifos inhibited the growth of wheat. Elevated CO_2 stimulated root growth more than shoot growth. Elevated CO_2 stimulated shoot biomass by a factor of 1.23 than ambient CO_2. Similarly, elevated CO_2 enhanced the root biomass by a factor of 2.52 than ambient CO_2. Effect of biochar on plant growth parameters varied from 1% to 38% than no biochar. Contrastingly chlorpyrifos inhibited plant growth parameters (both root biomass and shoot biomass) by 17% to 42%.

One way ANOVA revealed that the factors (CO_2, biochar, and chlorpyrifos) significantly ($p < 0.01$) influenced the parameters (nitrification rate, shoot biomass, root biomass, abundance of eubacteria and abundance of ammonia oxidizers) (Table 3). The interaction of CO_2 and chlorpyrifos (CO_2 × chlorpyrifos) significantly influenced the nitrification rate ($p < 0.001$), abundance of eubacteria ($p < 0$) and ammonia oxidizers ($p < 0.001$). The interaction of biochar and chlorpyrifos (biochar × chlorpyrifos) significantly influenced root biomass ($p < 0.01$) and abundance of ammonia oxidizers ($p < 0.001$). The interaction among CO_2, biochar and chlorpyrifos (CO_2 × biochar × chlorpyrifos) had no significant effect on any of the

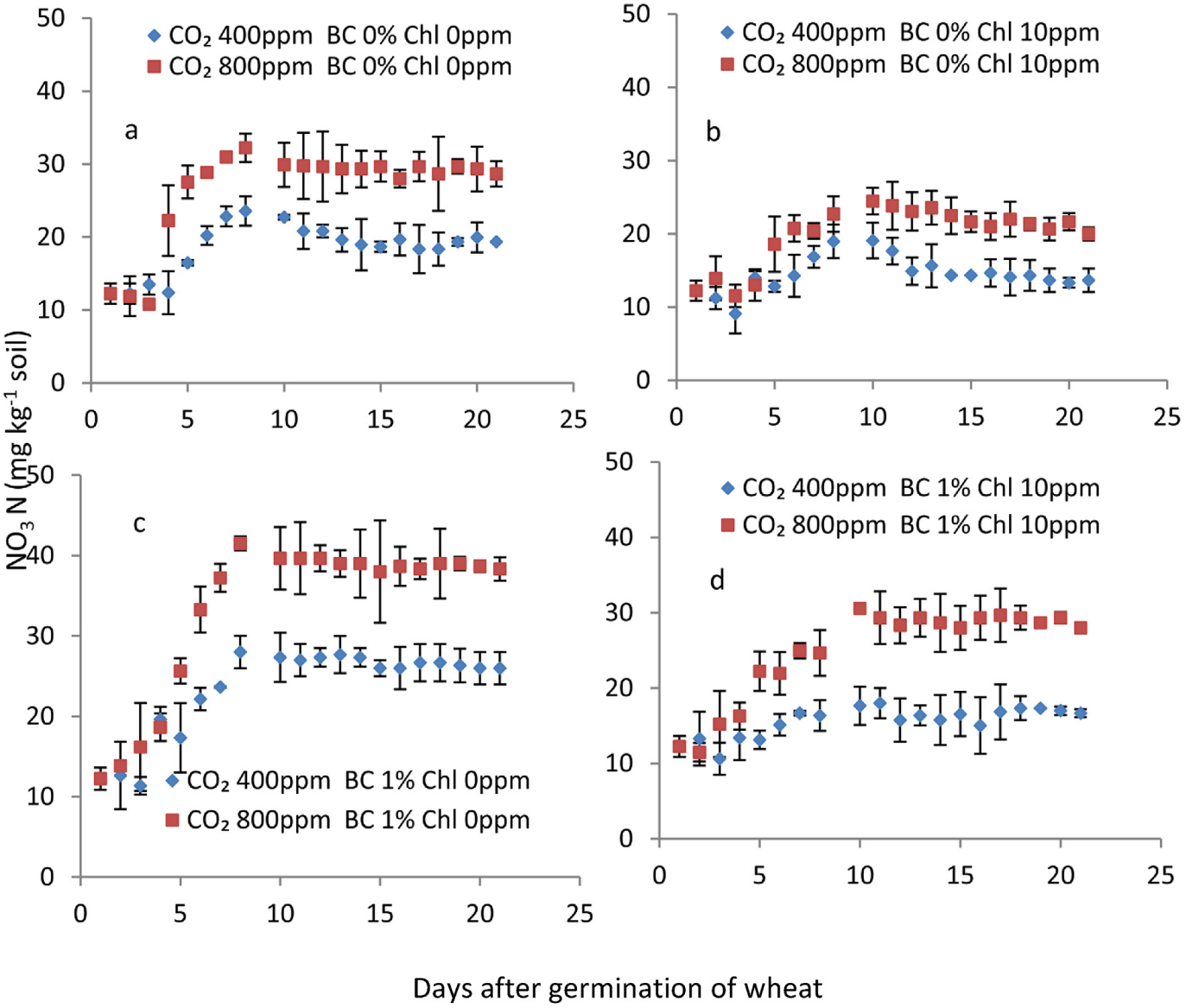

Fig. 1. Influence of CO_2, biochar (BC) and chlorpyrifos (Chl) on nitrification. NO_3 concentration in the rhizopheric soils of wheat was estimated. Wheat plants were grown under controlled environment with different CO_2 concentration (400 ppm, 800 ppm), biochar (0%, 1%), and chlorpyrifos (0 ppm, 10 ppm). NH_4-N (NH_4SO_4) was applied to soil at 100 kg N ha^{-1} basis. Panels are a: CO_2 with BC 0% with chlorpyrifos 0ppm, b: CO_2 with BC 0% with chlorpyrifos 10ppm, c: CO_2 with BC 1% chlorpyrifos 0 ppm, and d: CO_2 with BC 1% chlorpyrifos 10 ppm. Each data point represents arithmetic mean and standard deviation as error bar of three replicated observations. X axis represents incubation period and Y axis represents NO_3 concentration.

Table 1
Apparent nitrification rate (mg NO_3 N produced g^{-1} soil d^{-1}), abundance of 16S rRNA gene copies of eubacteria, amoA gene copies of nitrifying bacteria. Soils were amended with chlorpyrifos (0 ppm, 10 ppm), biochar (0%, 1%), and incubated under CO_2 (400 ppm, 800 ppm). Soils were planted with wheat. Each data represents arithmetic mean ± standard deviation of three replicated observations.

CO_2 (ppm)	Biochar (%)	Chlorpyrifos (ppm)	Nitrification rate (mg NO_3-N produced g^{-1} soil d^{-1})	16S rRNA gene (x 10^6 cells g^{-1} soil)	amoA gene of nitrifying bacteria (x 10^5 cells g^{-1} soil)
400	0	0	1.808 ± 0.276^{e}	34 ± 4.04^{e}	3.33 ± 0.577^{ef}
		10	0.665 ± 0.152^{h}	25 ± 3.00^{g}	1.43 ± 0.404^{g}
	1	0	2.368 ± 0.187^{c}	39 ± 3.78^{d}	3.66 ± 0.577^{e}
		10	0.760 ± 0.069^{g}	30 ± 1.73^{f}	3.03 ± 0.058^{f}
800	0	0	3.815 ± 0.407^{b}	74 ± 2.51^{a}	7.83 ± 0.764^{b}
		10	1.562 ± 0.293f	44 ± 5.29^{c}	4.33 ± 0.577^{d}
	1	0	4.525 ± 0.585^{a}	75 ± 4.04^{a}	8.33 ± 0.577^{a}
		10	2.157 ± 0.100^{d}	54 ± 4.04^{b}	6.06 ± 0.902^{c}
Tukeys HSD (p 0.05, df error 23)			0.061	1.88	0.076

Table 2
Wheat growth under the influence of CO_2, biochar and chlorpyrifos. Soils were amended with chlorpyrifos (0 ppm, 10 ppm), biochar (0%, 1%), and incubated under CO_2 (400 ppm, 800 ppm). Soils were planted with wheat and the growth of plants was measured as shoot biomass (g) and root biomass (g) after 45 days of sowing. Each data represents arithmetic mean ± standard deviation of three replicated observations. Mean values followed by same letters are not significantly different at $p < 0.05$.

CO_2 (ppm)	Biochar (%)	Chlorpyrifos (ppm)	Shoot biomass (g)	Root biomass (g)
400	0	0	27.6 ± 1.637^{e}	12.6 ± 0.346^{de}
		10	23.0 ± 2.000^{f}	9.6 ± 0.577^{f}
	1	0	30.7 ± 0.755^{d}	17.3 ± 1.429^{c}
		10	23.3 ± 1.155^{f}	10.0 ± 0.001^{e}
800	0	0	41.6 ± 2.887^{b}	21.6 ± 0.577^{b}
		10	29.3 ± 5.038^{d}	14.6 ± 3.055^{d}
	1	0	43.0 ± 3.700^{a}	25.4 ± 2.335^{a}
		10	34.3 ± 0.577^{c}	16.3 ± 2.309^{c}
Tukeys HSD (p 0.05, df error 23)			1.12	1.58

parameters. Linear regression models indicated that nitrification rate was linearly fitted with the shoot biomass, root biomass, abundance of eubacteria and ammonia oxidizers (Fig. 2). Shoot biomass fitted linearly as 5.428 × nitrification rate +19.64. Root biomass fitted linearly as nitrification rate × 3.914 + 7.332. Abundance of eubacteria linearly fitted as nitrification rate × 13.10 + 18.23. Similarly, the abundance of ammonia oxidizers fitted as nitrification × 1.608 + 1.207. The relative level of association among the variables and factors was evaluated by PCA. The ordination biplot categorized CO2 concentration and biochar as the most important principal components. The principal component (PC) 1 contributed approximately 93.28% and PC2 contributed 4.40% variance (Fig. 3). The parameters (nitrification rate, abundance of 16S rRNA gene copies, amoA gene copies, shoot biomass and root biomass) were positioned in the coordinates of elevated CO_2, indicating greater impact of CO_2 on the parameters. The vector of nitrification rate was closely placed with root biomass and amoA gene copies.

4. Discussion

The concentration of NO_3-N increased steadily soon after the addition of NH_4-N. The concentration of NO_3-N peaked during 10–12 days. This indicated that the soils were active and had high abundance of microbial groups involved in nitrification. Subsequently the concentration of NO_3-N remained at a steady state in the soil samples. Probably, the soils attained the maximum nitrification potential and resulted plateau level of NO_3-N. The wheat plant's N uptake is about 20–50 kg ha^{-1} at each of the three growth phases: maximum tillering, stem elongation and flowering stages (McGuire et al., 1998). In addition to the plant's NO_3-N uptake, soil microorganisms also assimilate considerable amount of NO_3-N. In soil ecosystem microbes assimilate both NH_4-N and NO_3-N. Heterotrophic microbes assimilate less NH_4^+ than NO_3^- because of competition by nitrifiers (Burger and Jackson, 2003). Therefore, the uptake of NO_3-N both by plant and microbes caused a saturation curve in the nitrification. It was also observed that out of 50 mg NH_4-N kg^{-1} only 30–66% was nitrified. This could be due to the effect of different factors on the nitrifiers and variation in N uptake by plant and microorganisms.

In general the elevated CO_2 exerted high impact compared to chlorpyrifos and biochar. Elevated CO_2 and biochar exhibited positive impact on the parameters while the chlorpyrifos exerted negative impact. For example, the positive impact of elevated CO_2 on nitrification was twice than that of the ambient CO_2. Elevated CO_2 stimulated root biomass and shoot biomass. Elevated CO_2 stimulated photosynthesis resulting net primary productivity. High root biomass improves root exudation leading to high microbial abundance than that of low root biomass. Under such scenario, the high microbial abundance probably stimulated the chlorpyrifos degradation alleviating toxic effect of chlorpyrifos on nitrification. Similar result has been observed in a tropical rice soil. In a rice field experiment biodegradation of chlorpyrifos under the influence of elevated CO_2 was explored. About 88.4% of initially applied chlorpyrifos was degraded from the rice soil maintained under elevated CO_2 (700 ppm) by 5 day, whereas the 80.7% of the chlorpyrifos was degraded under ambient CO_2 (Adak et al., 2016). Thus the elevated CO_2 reduced the negative impact of chlorpyrifos and stimulated nitrification. Secondly, elevated CO_2 increased microbial abundance including nitrifiers which in turn stimulated nitrification. Positive effect of elevated CO_2 on nitrification can be explained by two means based on the microbial abundance and plant growth. Nitrifiers are autotrophs and obligatory aerobic in nature. Nitrifiers use CO_2 as substrate for their growth and metabolism. Therefore, elevated CO_2 stimulated nitrifying microbial population. Elevated CO_2 increased the plant biomass as well as root biomass. This effect was due to the CO_2-C fertilization. High growth of plant biomass also causes high root exudation as discussed before. In a free air carbon dioxide (CO_2) enrichment (FACE) field experiment Spring wheat (*Triticum aestivum* L. cv. TRISO) was grown for three consecutive seasons in order to examine the effects on crop yield and grain quality. Elevated CO_2 promoted the aboveground biomass (+11.8%) and grain yield (+10.4%) (Högy et al., 2009). In another experiment the effect of elevated CO_2 on the root structure and function and agronomic properties of two wheat (*Triticum aestivum* L.) cultivars was studied. Elevated CO_2 improved the N uptake and enhanced the allocation of N to grains (Tausz et al., 2017). Elevated CO_2 influences microbial structure and function through increased root exudation in plants (Wang et al., 2017). High soil organic carbon due to increased root exudation stimulated microbial activity (nitrification) and aided in plant's N uptake.

Biochar stimulated the nitrification potential of soil irrespective of other factors. In a study it was reported that biochar promoted soil ammonia-oxidizer populations (bacterial and archaeal nitrifiers) and accelerated gross nitrification rates more than two-fold (Prommer et al., 2014). The positive effect of biochar is linked to its properties. Biochar is a porous material and has high surface area (Atkinson et al., 2010). Both the physical properties favour the growth of aerobic microorganisms. Ammonia oxidizing bacteria are obligate aerobes and were

Table 3
Analysis of variance (ANOVA) of the factors and variables to determine interaction effect of CO_2, biochar and chlorpyrifos on apparent nitrification rate, wheat shoot biomass, root biomass, population of eubacteria (16S rRNA gene copies), and ammonia oxidizing bacteria (AOB) in soil.

Factors	Nitrification	Shoot	Root	Eubacteria	AOB
CO_2 (C)	< 0.0001***	< 0.0001***	< 0.0001***	< 0.0001***	< 0.0001***
Biochar (BC)	0.00117**	0.0374*	0.00149**	0.00279**	0.00532**
Chlorpyrifos (Chl)	< 0.0001***	< 0.0001***	< 0.0001***	< 0.0001***	< 0.0001***
C x BC	0.20990	0.5032	0.90087	0.82825	0.48649
C x Chl	0.00172**	0.0518.	0.05163.	< 0.0001***	0.00461**
BC x Chl	0.26191	0.8498	0.03007*	0.14221	0.00260**
C x BC x Chl	0.49171	0.1610	0.41457	0.14221	0.33988

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

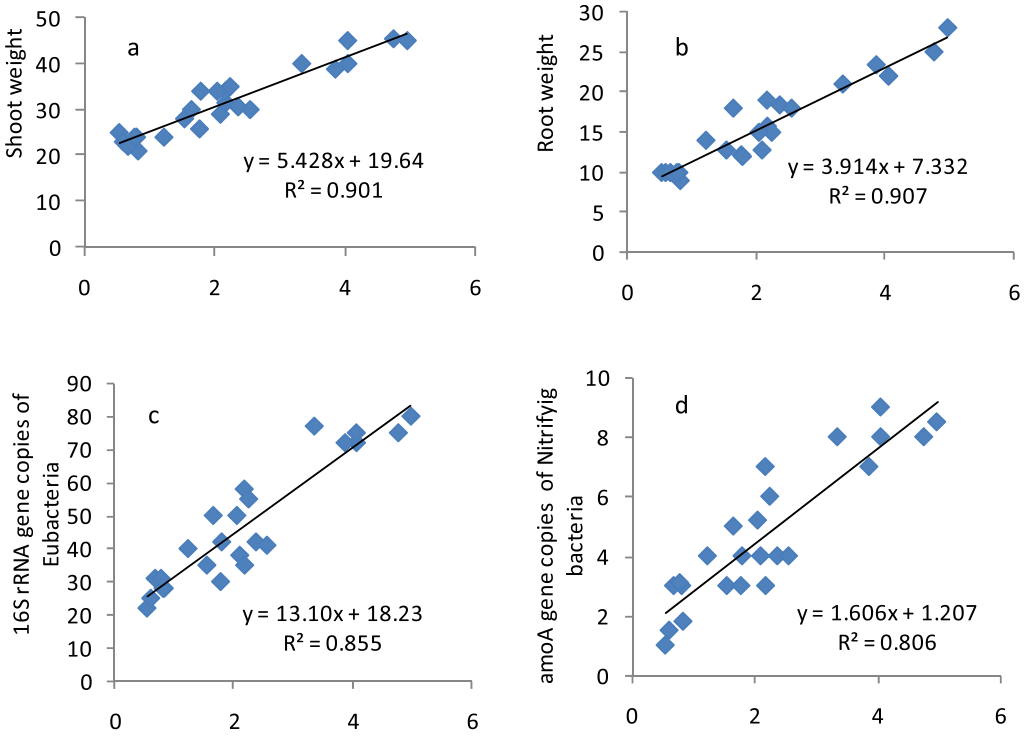

Fig. 2. Linear regression models (α 0.05) of nitrification and different parameters of the study. The parameters were fresh biomass (weight) of shoot (a), fresh biomass (weight) of root (b), abundance of 16S rRNA gene of eubacteria (c), and abundance of amoA genes of ammonia oxidizing bacteria (d). Y axis represents different parameters while the X axis represent nitrification rate.

thus stimulated in the soil amended with biochar. Biochar also provides shelter to microorganisms within its pores. Therefore, the abundance of microorganisms (both heterotrophic bacteria and ammonia oxidizers) was significantly ($p < 0.05$) high in the biochar amended soils. Chemically biochar is rich in minerals (Zhao et al., 2013). Ammonia oxidizers are chemolithoautotrophs and possibly the inorganic minerals favoured nitrification. Biochar has high cation exchange capacity (CEC) (Steinbeiss et al., 2009). High CEC allows adsorption of positively charged molecules like NH_4+. Higher availability of NH_4 favoured the ammonia oxidizers residing in the pores of biochar. Biochar also stimulates the decomposition of chlorpyrifos. Biochar has been found to positively stimulate biodegradation of several xenobiotics. In this study biochar stimulated the population of heterotrophic bacteria. Heterotrophic bacteria like *Acenetobacter*, *Enterobacter* biodegrade the organic pollutants including chlorpyrifos (Singh et al., 2004). Biochar has been reported to stimulate CH_4 oxidation by enhancing the abundance of aerobic methanotrophs, heterotrophs and actinomycetes (Ahirwar et al., 2018). Methanotrophs also carry out nitrification and possibly enhanced the nirification (Kollah et al., 2015).

The concentration of chlorpyrifos was within the range of recommended field dose of field application (Posey et al., 2006). Abundance of eubacteria and ammonia oxidizers was estimated at the peak nitrification period. Chlorpyrifos inhibited the abundance of eubacteria and nitrifiers. In an earlier study it was observed that chlorpyrifos inhibited methanotrophs (Ahirwar et al., 2018). Chlorpyrifos inhibits bacterial enzymes including hydrolases (carboxylesterase, acid phosphatase, β-glucosidase, urease and protease) and oxidoreductases (dehydrogenase and catalase) (Sanchez-Hernandez et al., 2017). Heterotrophic bacterial population represent the overall microbial activity of a soil and many heterotrophs degrade chlorpyrifos (Singh et al., 2004). Change in the abundance of both eubacteria and nitrifiers varied concurrently with the nitrification potential. Probably, the favourable factors (biochar and elevated CO_2) and stress factors (chlorpyrifos) influenced bacterial abundance parallel to nitrification.

One way ANOVA revealed that the factors (CO_2, biochar and chlorpyrifos) individually as well as interactively influenced ($p < 0.01$) the parameters (nitrification, microbial abundance and plant growth). This was due to the significant effect of the factors on parameters. However, the interaction of factors did not exhibit significant influence on the parameters. Wheat shoot and root biomass exhibited significant ($p < 0.01$) positive relation with the nitrification rate. PCA was self explanatory to reveal the complex interaction among factors and variables. PCA biplot identified CO_2 as the primary factor influencing the parameters. Nitrification rate was closely related to root biomass and shoot biomass and amoA gene copies. The negative effect of chlorpyrifos on wheat growth could be due to the inhibition of soil bacteria (both heterotrophic eubacteria and nitrifiers). The negative effect of chlorpyrifos was negated by biochar. Biochar stimulates degradation of many insecticides due to its high CEC, porosity and carbonaceous nature as discussed above (Khorram et al., 2016). Biochar also adsorbs insecticides and reduces bioavailability for plant uptake and enhanced plant growth (Yu et al., 2009). Biochar also absorbs insecticides and channelize to the inhabiting microbes for degradation (Jing et al., 2018). Considering these facts it is postulated that biochar can be used to manage chlorpyrifos contamination and improve crop growth under climate changing condition.

5. Conclusion

Based on this experiment it can be concluded that the negative effect of chlorpyrifos on nitrification can be subsidized by elevated CO_2 and biochar addition. The elevated CO_2 may act as C fertilizer and increased root and shoot biomass. Probably, the high root exudates due to increased plant root biomass stimulated the eubacteria and ammonia oxidizing bacteria and nitification. Biochar was effective in regulating the negative impact of chlorpyifos due to its physico chemical nature.

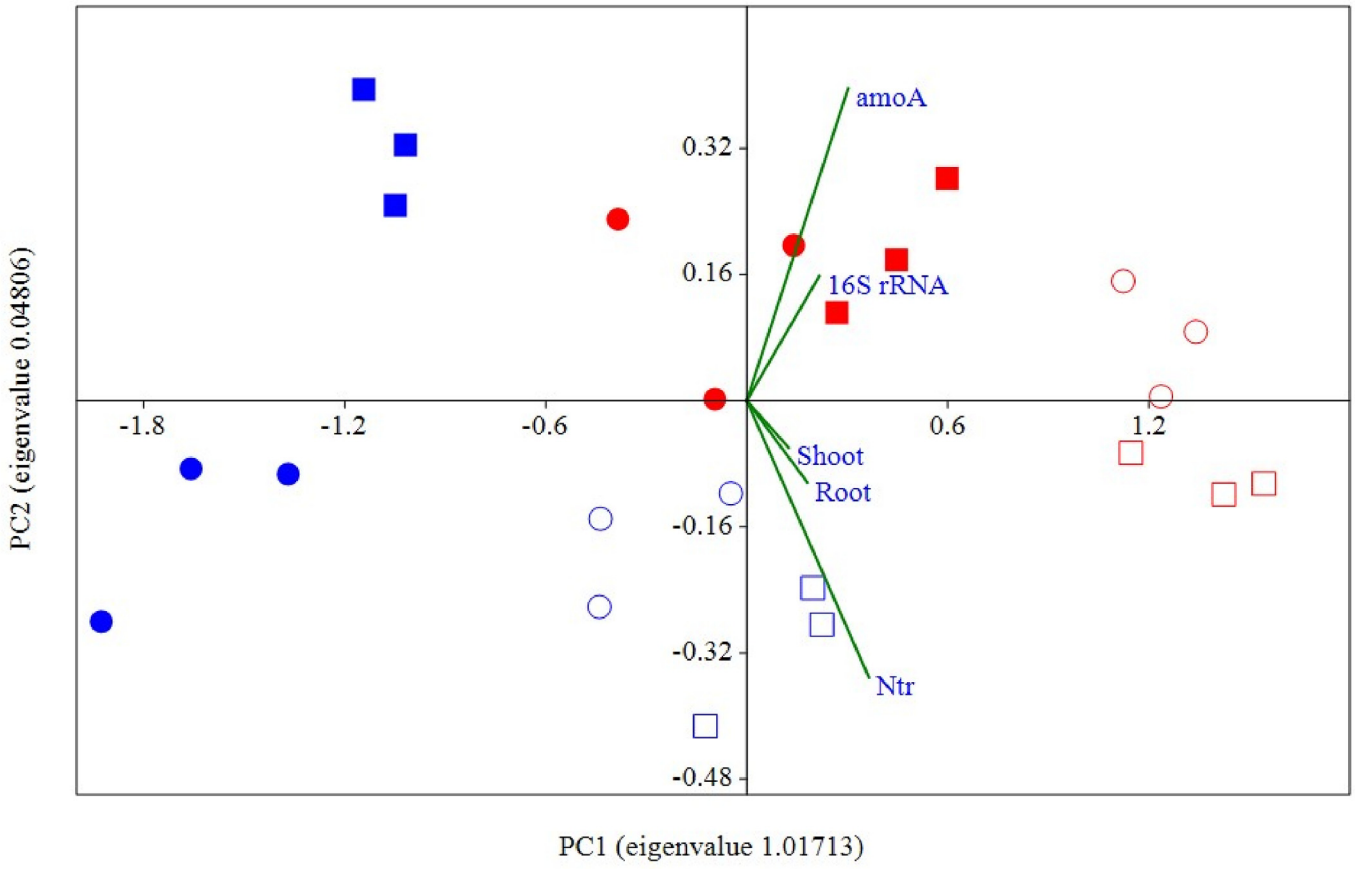

Fig. 3. Ordination biplot of principal component analysis (PCA) with component 1 and 2. Vectors of variables (lines) were nitrification rates (Ntr), abundance of eubacterial 16S rRNA gene copies (16S rRNA), abundance of ammonia oxidizing bacterial amoA gene copies (amoA), root biomass (Root) and shoot biomass (Shoot). Vectors of factors (symbols) were ambient CO_2 concentration, biochar, and chlorpyrifos. Treatments are presented as follows: ambient (400 ppm) CO_2 biochar 0% chlorpyrifos 0ppm (blue open circle), ambient CO_2 biochar 0% chlorpyrifos 10 ppm (blue filled circle), ambient CO_2 biochar 1% chlorpyrifos 0 ppm (blue open square), ambient CO_2 biochar 1% chlorpyrifos 10 ppm (blue filled square), elevated (800 ppm) CO_2 biochar 0% chlorpyrifos 0ppm (red open circle), elevated CO_2 biochar 0% chlorpyrifos 10 ppm (red filled circle), elevated CO_2 biochar 1% chlorpyrifos 0 ppm (red open square), elevated CO_2 biochar 1% chlorpyrifos 10 ppm (red filled square). For each treatment values are presented in three replicated observations. In PCA, arrows with narrow angles are strongly correlated, arrows that are perpendicular show no correlation and arrows in opposite directions indicate negative correlation.

As predicted by IPCC the increasing atmospheric CO_2 is likely to have positive effect on nitrification in the rhizosphere of wheat. However, there is need of experiments considering the effect of rising temperature for a holistic understanding on the interaction of climate, biochar and chlorpyrifos on nitrification.

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